Transcript Expression of FRNK Reveals Stage-Specific Requirement for Focal Adhesion Kinase Activity in Cardiac Growth


Abstract—Focal adhesion kinase (FAK) is strongly activated by integrins and growth factors and is essential for embryonic development. We previously showed that the C terminus of FAK is expressed as a separate protein termed FAK-related nonkinase (FRNK) in a smooth muscle cell–selective fashion and that FRNK functions to buffer FAK-dependent signals. We now show that FRNK is also transiently expressed in the neonatal myocardium, with peak levels occurring 5 to 7 days postnatal, just before cell cycle withdrawal. Using novel mouse models, we demonstrate that cardiac-selective expression of FRNK (leading to inhibition of FAK) starting at embryonic day 10.5 leads to a severe ventricular noncompaction defect associated with reduced cardiomyocyte proliferation. Remarkably, postnatal expression of nearly identical levels of FRNK is well tolerated and does not affect viability or anabolic cardiac growth. Nonetheless, FRNK expression in the adult heart does attenuate pathological cardiac hypertrophy following aortic banding, confirming and extending our previous data that this compensatory response is blunted in FAK null hearts. Our mechanistic studies in cultured neonatal cardiomyocytes reveal that FRNK expression induces p38/p27kip-dependent cell cycle withdrawal and attenuates extracellular signal-regulated kinase–dependent hypertrophic growth. These findings indicate that dynamic expression of FRNK in the neonatal heart may function to promote cardiomyocyte quiescence in an environment that is particularly rich in growth factors and growth promoting extracellular matrices. (Circ Res. 2009;104:1201-1208.)

Key Words: cardiomyocyte ■ cell cycle ■ focal adhesion kinase ■ ventricular noncompaction

Robust, locally restricted myocyte proliferation is necessary for several stages of cardiac morphogenesis, and, although it has long been known that cardiomyocytes undergo terminal differentiation and exit from the cell cycle shortly after birth, the mechanisms that regulate fetal myocyte cell cycle progression and those that promote cell cycle withdrawal are not well understood.

The nonreceptor tyrosine kinase focal adhesion kinase (FAK) is strongly activated by both integrins and growth factors and is a likely candidate to integrate signals from these diverse pathways during growth and development.1 Germline deletion of FAK results in general mesodermal defects and embryonic lethality between embryonic day (E)8.5 to E10, and examination of the malformed hearts from these embryos indicated a putative function for FAK in cardiomyocyte maturation.2 However, because FAK can serve both structural and signaling roles within focal adhesions, it has been difficult to determine whether FAK activity per se is essential for regulating cardiomyocyte growth. Interestingly, our laboratory recently showed that FAK activity can be regulated by the endogenous expression of a separate protein comprising the carboxyl terminus of FAK, termed FRNK (FAK-related nonkinase) that acts as a dominant interfering mutant ing the carboxyl terminus of FAK.3 FRNK transcription results from the utilization of an alternative start site within the FAK gene, and FRNK expression is independently regulated by a distinct promoter embedded within FAK intronic sequences.4,5 Whereas FAK expression is ubiquitous relatively constantly during development, we previously found that FRNK protein was particularly high in the vasculature when smooth muscle cells (SMCs) are transitioning from a synthetic to contractile phenotype.4

We now present evidence that FRNK is also transiently expressed in the neonatal myocardium before cell cycle withdrawal. By conditionally expressing FRNK in the fetal and neonatal myocardium in the mouse, we show that FAK activity mediates both hyperplastic and pathological hypertrophic growth but not anabolic growth of the heart. Our findings lead us to propose that dynamic expression of FRNK in the neonatal heart may function to dampen cardiomyocyte cell cycle progression (and to facilitate cell cycle withdrawal)
in an environment that is particularly rich in growth factors and growth-promoting extracellular matrix (ECM).

Materials and Methods

Transgenic mice were generated and backcrossed to the C57black6 background. Mice were housed in the University of North Carolina Animal Care Facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and all experimental procedures were approved by the University of North Carolina Animal Care and Use Committee. See the expanded Materials and Methods section in the online data supplement at http://circres.ahajournals.org for a complete description of the reagents, DNA constructs, transgenic mouse generation, and general methods used for these studies.

Statistical Analysis

All quantitative data represent at least 3 separate experiments and are presented as means±SEM. Means were compared by 2-tailed Student’s t test. P<0.05 was considered statistically significant, as indicated by an asterisk. All other data including Western analysis, are representative of at least 3 individual experiments.

Results

Transient Expression of Cardiac FRNK

We previously reported that FRNK is dynamically and selectively expressed in SMCs during neonatal development and following vascular injury in adult rodents.4 However, during the course of our previous examination of FAK signaling in the developing myocardium,6 we made the somewhat surprising observation that FRNK is also transiently expressed in the neonatal myocardium. As shown in Figure 1a, peak FRNK protein levels were observed between postnatal day (P)5 and P7, whereas high levels of FAK were observed in the myocardium from E10.5 to P7. During heart development, 2 forms of FRNK (with apparent molecular masses of 41 and 43 kDa) appear to be present, consistent with our previous findings in SMCs.4 (See the online data supplement for further discussion.) We next isolated cardiomyocytes from neonatal rat hearts and performed Western analysis to ensure that FRNK was selectively expressed in these cells. As shown in Figure 1b, expression of FRNK in purified cardiomyocytes mirrored the levels found in the intact myocardium, with high levels observed in isolated P7 relative to P0 cardiomyocytes. The temporal expression of FRNK in neonatal cardiomyocytes highlights the possibility that precise control of FAK signaling might be necessary for proper growth of the heart.

FRNK transcription results from the utilization of an alternative promoter embedded between exons encoding the FAK-kinase domain and the FAK carboxyl-terminal protein-binding region,5 and we showed that FRNK-specific mRNAs are initiated within a unique 5′ noncoding exon in vascular SMCs and smooth muscle–containing tissues.4 However, data also indicate that FRNK-like peptides can be generated in cells by protease degradation of FAK.7,8 To discriminate between these 2 possibilities, we subjected ventricular myocardial RNAs (isolated from the apex of the heart) to quantitative RT-PCR using probes and primers directed against the FRNK noncoding exon. As shown in Figure 1c, FRNK mRNA was apparent in ventricular myocardial tissue, and the levels peaked between P1 and P4, correlating well with FRNK protein patterns detected by immunoblot analysis. This transient expression of FRNK in the early postnatal heart coincides with the window in which cardiomyocytes transition to terminally differentiated cells. Because our previous data indicated that FRNK functions to buffer of FAK-dependent growth signals in SMCs,9 we reasoned that FRNK might function to decrease DNA synthesis and possibly promote cell cycle withdrawal in cardiomyocytes.

Generation of FRNKloxp Mice

To carefully examine the function of FRNK in the myocardium in vivo, we generated a Cre-dependent transgenic mouse model in which FRNK can be overexpressed in a time- and tissue-restricted fashion (Figure 2A; see the expanded Results section in the online data supplement for description). Western blot analysis revealed moderate levels of green fluorescent protein (GFP) expression in all tissues examined from 2 independent transgenic lines of these so-named FRNKloxp mice, whereas myc-FRNK was undetectable in these lysates (Figure I in the online data supplement). Immunohistochemistry for GFP revealed uniform expression throughout the heart (Figure 2d) and several other tissues as expected (data not shown).

Targeted Expression of FRNK in the Myocardium

By crossing the FRNKloxp mice to well-established Nkx2.5Cre and MLC2vCre lines, we were able to explore the functional consequences of FRNK expression early and late during cardiac development.10,11 FRNKloxp/MLC2vCre double transgenic mice (hereafter referred to as FRNKMic2v) were born in the expected Mendelian ratios, and Western blot analysis revealed cardiac-restricted expression of myc-FRNK from P1 onwards (Figure 2b and 2c and Online Figure II). Moreover, only a few GFP-positive cells were detected by immunohistochemistry in the ventricles of the FRNKMic2v hearts, indicating nearly complete transgene recombination (Figure 2d).
Importantly, hearts from FRNK\textsuperscript{Mic2v} mice exhibited decreased FAK activity relative to hearts from control FRNK\textsuperscript{loxP} mice, as determined by Western blotting with an anti–pFAK Y-397 antibody (Figure 2e). Nonetheless, FRNK\textsuperscript{Mic2v} mice did not exhibit any overt abnormalities and had normal life expectancies (Figure 2f and Online Table I).

In stark contrast, crossing FRNK\textsuperscript{loxP} mice with the Nkx2.5\textsuperscript{Cre} line yielded no viable double transgenics, and subsequent studies revealed that embryonic lethality in FRNK\textsuperscript{Nkx2.5} mice occurred between E14.5 and E16.5 (Figure 2f and Online Table I). Western analysis revealed detectable levels of myc-FRNK as early as E10.5 in FRNK\textsuperscript{Nkx2.5} hearts (data not shown), with maximal levels observed by E13.5 (Figure 2g). Importantly, although the timing of myc-FRNK expression was different between the 2 models, the maximal levels of myc-FRNK protein expressed in the hearts of FRNK\textsuperscript{Nkx2.5} and the FRNK\textsuperscript{Mic2v} mice were remarkably similar and modestly (3-fold) higher than levels of endogenous FRNK expressed in control hearts at P7 (see Online Figure II for quantification). Collectively, these data indicate that embryonic cardiac development is exquisitely sensitive to FRNK expression (and a reduction in FAK activity), whereas further postnatal development is FAK-independent.

Histological and Morphological Analysis of FRNK\textsuperscript{Nkx2.5} Mice

Gross morphology of FRNK\textsuperscript{Nkx2.5} embryos at E14.5 revealed extreme hydrops fetalis and blood pooling, likely attributable to cardiac failure in these embryos (Figure 3a). Histological examination of coronal sections through the FRNK\textsuperscript{Nkx2.5} hearts revealed similar heart size and ventricular wall thickness at E10.5 (the onset of myc-FRNK expression). However, as development progressed from E10.5 to E14.5, little growth was observed in the FRNK\textsuperscript{Nkx2.5} hearts in comparison to genetic controls, which underwent dramatic expansion of the ventricular walls and growth of the interventricular septum (Figure 3b). Whereas trabeculae were reasonably well formed in the FRNK\textsuperscript{Nkx2.5} hearts, compaction of the ventricular chambers was significantly impaired. Indeed, quantification of E14.5 coronal heart sections revealed a 48% reduction in cell density in the FRNK\textsuperscript{Nkx2.5} hearts compared to age-matched controls (Figure 3c). There were no overall gross or histological differences observed in other tissues derived from nknk2.5-expressing cells compared to genetic controls at E14 (Online Figure IV and Online Results), indicating that lethality in FRNK\textsuperscript{Nkx2.5} embryos was likely attributable to heart failure at approximately E15.5.
myc-FRNK Expression Attenuates Myocyte Proliferation

Because no significant differences were observed in the cardiac differentiation program or the extent of apoptosis between E13.5 FRNK\textsuperscript{Nkx2.5} and genetic control hearts (Figure 3d and Online Results), we next sought to determine whether FRNK expression led to a proliferation defect. Indeed the number of 5-bromodeoxyuridine (BrdUrd)-labeled cells in the ventricles of E13.5 FRNK\textsuperscript{Nkx2.5} hearts was significantly reduced when compared to genetic control hearts (especially within the compact zone; Figure 4). We did not observe significant differences in BrdUrd incorporation in any other tissues examined (data not shown). We next turned to cultured primary cardiomyocytes to determine whether FRNK expression could directly impair proliferation of neonatal cardiomyocytes. To this end, we infected P0 cardiomyocytes with GFP or GFP-FRNK adenovirus and examined the rate of proliferation of these cells in serum-containing media. Under these conditions the rate of BrdUrd incorporation in GFP-infected cells was \textasciitilde18\%, whereas only 4\% of the FRNK-infected cells were BrdUrd-positive (Figure 5a and 5f). In addition to this striking reduction in BrdUrd incorporation, the FRNK-infected cells exhibit a blunted hypertrophic response to serum, as assessed by reduced cell size and sarcomeric actin organization (as assessed by cardiac troponin T staining; see Online Figure VIII, a, for higher-power images), consistent with our previous findings.\textsuperscript{12} Collectively, these data indicate that FRNK expression negatively regulates embryonic and neonatal cardiomyocyte growth but does not affect cell survival during postnatal heart development.

myc-FRNK Expression Regulates Mitogen-Activated Protein Kinase Signaling

Previous studies have revealed that the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated ki-
nase (ERK) and p38 play positive and negative roles, respectively, in regulating cardiomyocyte cell cycle progression.13–17 Interestingly, we found that ERK activity (Figure 5b) and expression of the ERK-dependent immediate early gene c-fos (Online Figure V, b) were each significantly reduced in E13.5 FRNK\textsuperscript{Knx2.5} hearts compared to littermate controls at this time point (the peak of myocyte proliferation). In contrast, p38 activity was significantly elevated in FRNK\textsuperscript{Knx2.5} hearts (Figure 5b). To confirm that regulation of these MAPKs was attributable to a primary change in FRNK expression, we examined activation of these pathways in cultured cardiomyocytes. Indeed, ectopic expression of FRNK significantly enhanced p38 activity in a time-dependent fashion but attenuated growth factor (fibroblast growth factor [FGF]-2 and insulin-like growth factor [IGF]-1)–induced ERK activity in these cells (Figures 5c and 6b; see Online Figure VI for quantification).

To determine the functional importance of these pathways with respect to FRNK-dependent inhibition of myocyte proliferation, we explored whether inhibition of p38 or activation of ERK would restore myocyte proliferation in FRNK-expressing cardiomyocytes. As shown in Figure 5a and 5f, treatment with the p38 inhibitor SB203580 (10 \textmu mol/L) enhanced BrdUrd incorporation in GFP-expressing cells (by 2-fold) and even more markedly enhanced BrdUrd incorporation in FRNK-expressing cells by 5-fold (to 25%; surpassing the level observed in GFP control cells). With respect to ERK activation, we found that treatment of GFP-expressing cardiomyocytes with the MEK1 inhibitor UO927 reduced BrdUrd incorporation by \approx 50\%, indicating that ERK activation is necessary for maximal serum-stimulated BrdUrd incorporation. However, adenoviral-mediated expression of constitutively active MEK1, which led to sustained elevated ERK activity (see Online Figure VIII, b), induced only a modest increase in BrdUrd incorporation in both GFP- and FRNK-infected cells, and no synergy was found on treatment with constitutively active MEK and the p38 inhibitor (Figure 5f; see Online Figure VIII, a, for representative images). Importantly, FRNK was expressed in comparable levels under all treatment groups, as assessed by Western analysis (Online Figure VIII, b).

We next evaluated the expression levels of the cell cycle inhibitor p27\textsuperscript{kip} (a key p38-dependent cell cycle regulator) in GFP- and FRNK-expressing cardiomyocytes. As shown in Figure 5e, expression of p27\textsuperscript{kip} was markedly upregulated in FRNK-expressing cells in comparison to the GFP-expressing controls. Notably, treatment of the FRNK-expressing cells with SB203580 restored expression of p27\textsuperscript{kip} to control levels, p27\textsuperscript{kip} levels were also significantly elevated in E13.5 FRNK\textsuperscript{Knx2.5} hearts in comparison to genetic controls (Figure 5b). Collectively, these data indicate that FRNK expression attenuates cardiomyocyte cell cycle progression and induces cell cycle withdrawal, in large part, by a mechanism that involves enhanced activation of p38, an important negative regulator of cardiomyocyte cytokinesis.16,17 Interestingly, we also observed upregulation of p38 activity in FAK-null hearts (see Online Results and Online Figure VII), indicating that FRNK expression likely dampens cardiomyocyte proliferation by relieving FAK-dependent repression of p38.

FRNK\textsuperscript{Mlc2v} Mice Exhibit Normal Anabolic Growth but a Diminished Response to Pressure Overload
Because FAK activity was clearly necessary for growth of the fetal heart, we carefully assessed the affect of FRNK expression on postnatal myocardial growth. After extensive measurements of heart size, heart weight, cardiomyocyte cross-sectional area, myofibrillar organization, collagen deposition, and physiological function (by echocardiography), we concluded that there were no significant differences in hearts from FRNK\textsuperscript{Loxp} and FRNK\textsuperscript{Mlc2v} mice up to 8 months of age (Figure 6a and Online Table II). These data reveal that FRNK expression is well tolerated in terminally differentiated myocytes and imply that FAK activity is not necessary for anabolic growth of the heart.

Postnatal anabolic (physiological) hypertrophic growth is known to be controlled primarily by IGF-1– and growth hormone–dependent stimulation of the phosphatidylinositol 3-kinase/AKT/p70S6K protein synthesis pathway.18–23 Interestingly, we found that whereas IGF-1–stimulated (and FGF-
stimulated) ERK activation was attenuated by FRNK expression in neonatal cardiomyocytes, IGF-1–stimulated activation of AKT was not (Figure 6b). Moreover, IGF-1 stimulation led to a comparable induction of AKT in cardiomyocytes plated on either fibronectin (which promotes β1 integrin–dependent FAK activation) or on type I collagen (which does not promote FAK activation; Figure 6c), whereas IGFI-stimulated (and FGF-stimulated) ERK activity required fibronectin (Figure 6d). Collectively, these data reveal that FRNK/FAK signaling modulates a subset of myocyte growth responses, effecting signals that regulate cell cycle progression but not anabolic growth.

Although FAK protein levels and basal activity are relatively low in the adult myocardium, several reports have indicated that FAK activity is markedly induced following mechanical overload, and we recently published a report showing that myocyte-restricted FAK deletion attenuates compensatory hypertrophic remodeling following pressure overload.24 However, questions remain as to whether FAK plays a structural role or signaling role in this setting. Thus, we next strove to determine whether specific inhibition of FAK activity in vivo in the FRNKMc2v line was sufficient to blunt pressure overload–induced hypertrophic remodeling. To this end, we subjected FRNKMc2v and wild-type littermate control mice to a minimally invasive aortic banding procedure that provides an approximate 50% reduction in the lumen of the ascending aorta.25 Following chronic aortic constriction, we examined the mice for a variety of hypertrophic changes. As shown in Figure 7a, FRNKMc2v mice had significantly lower heart weight versus body weight in comparison to genetic controls following banding. In addition, myocyte cross-sectional area was significantly lower in postbanded FRNKMc2v hearts compared to postbanded genetic controls (Figure 7b). Echocardiographic analysis of genetic control mice also revealed significant differences in the extent of remodeling following banding. Notably, the pressure overload–induced increase in thickness of the left ventricular wall and intraventricular septum were both significantly reduced in the FRNKMc2v hearts when compared to the genetic controls (Figure 7c). Hearts from FRNKMc2v and control mice were also analyzed for hypertrophic marker gene expression by quantitative RT-PCR before and after banding. Importantly, the fold increase in the canonical hypertrophic marker gene, atrial natriuretic factor was significantly attenuated in the FRNKMc2v hearts in comparison to the genetic controls (control, 66±10.5-fold increase in banded over sham; FRNK, 3.8±1.2-fold increase in banded over sham; P<0.05; n=6 per genotype).

As expected, adrenergic agonist-induced ERK activation was also dampened in FRNKMc2v hearts in comparison to those from genetic controls (Figure 7d). Interestingly, we noted in our proliferation studies that ectopic expression of Mek1 (but not inhibition of p38) reversed the hypertrophic growth defect in FRNK-expressing cells (see Figure 5a and Online Figure VIII, a). Subsequent studies revealed that expression of constitutively active Mek1 induced a 2-fold increase in cell area in FRNK-expressing cardiomyocytes and enhanced sarcomeric actin organization in these cells (a hallmark of pathological hypertrophic growth; Online Figure VIII, c and d).

Collectively, these data indicate that promotion of p38 activity and attenuation of ERK activity are likely the major mechanisms by which FRNK expression represses proliferation and pathological hypertrophic growth, respectively.

**Discussion**

It is clear that coordinated signaling through integrins and growth factors plays an important role in regulating both cardiac morphogenesis and the progression of cardiac disease, but how these processes are fine-tuned during the different phases of heart growth and development is unknown. Herein, we show that FRNK, the endogenous inhibitor of FAK, is transiently expressed in heart, with peak levels occurring at P5 to P7, when cardiomyocytes are transitioning to terminally differentiated cells. Using novel mouse models, we demonstrated that cardiac-selective expression of FRNK (leading to inhibition of FAK) starting at E10.5 led to a severe ventricular noncompaction defect associated with impaired cardiomyocyte proliferation. Remarkably, postnatal expression of nearly identical levels of FRNK was well tolerated and did not affect viability or anabolic cardiac growth but did attenuate pressure overload–induced hypertrophic remodeling. Collectively, our mechanistic studies indicate that FAK regulates p38-dependent myocyte proliferation in the developing myocardium and ERK-dependent promotion of patho-
logical hypertrophy following biomechanical stress but that FAK activity is dispensable for AKT-dependent physiological, anabolic growth of the heart.

Our recent demonstration that FAK activity is enhanced in tissues and cells derived from FRNK−/− mice strongly supports the hypothesis that the dynamic regulation of FRNK expression can impart specific spatial and temporal control of FAK activity in vivo.26 Additionally, a very recent publication revealed that myocyte-specific deletion of FAK in mid-gestation (using the Mlc2aCre line) phenocopies the defects we observed in the FRNKNkx2.5 embryos including embryonic lethality, global edema, thin ventricle wall, and reduced rates of myocyte proliferation, although the underlying signaling mechanisms were not explored.27 Thus, mice with cardiac-restricted expression of FRNK exhibit remarkably similar developmental and hypertrophic growth defects as mice with similar temporal deletion of FAK. Collectively, these studies strongly support the notion that the effects of FRNK are mediated by inhibition of FAK-dependent signals and that FAK activity is necessary to promote myocyte proliferation in a cell-autonomous fashion.

Proliferation of cardiomyocytes within the compact zone at mid-gestation is necessary to support the increasing hemodynamic load of the embryo, and a large body of evidence indicates that FGF-, IGF-, and transforming growth factor/bone morphogenetic protein–induced signals appear to play a critical role in this process because genetic deletion of these ligands or their receptors lead to compaction defects.15 In terms of signaling, many of these receptors are linked to the MAPK signaling pathways terminating in activation of ERK, c-Jun N-terminal kinase, and p38. Several recent studies indicate that p38 may function at a G2/M checkpoint to block cardiomyocyte cell cycle reentry.16,17 including the findings that (1) p38α activity is inversely correlated with cardiac growth during development; (2) activation of p38 in vivo (by forced expression of MKK3) reduced fetal cardiomyocyte growth; (3) cardiac-restricted deletion of p38α significantly enhanced myocyte proliferation; and (3) cardiac-restricted deletion of p38α significantly enhanced myocyte proliferation. Our studies reveal that expression of FRNK limits cardiomyocyte proliferation and induces cell cycle arrest in a p38-dependent fashion. Indeed, we found that expression of FRNK (or inactivation of FAK) promotes p38 activity and regulates expression of the p38-dependent cell cycle modifier, p27Kip1 and that pharmacological inhibition of p38 in vivo (by forced expression of MKK3) reduced fetal cardiomyocyte proliferation; and (3) cardiac-restricted deletion of p38α significantly enhanced myocyte proliferation. Our studies reveal that expression of FRNK limits cardiomyocyte proliferation and induces cell cycle arrest in a p38-dependent fashion.

Our studies reveal that the timing of FRNK expression in the postnatal heart corresponds to the window during which cardiomyocytes exit the cell cycle. (See the online data supplement for further discussion of FRNK expression in the myocardium.) Because misexpression of FRNK can attenuate proliferation of embryonic cardiomyocytes, we speculate that transient expression of endogenous FRNK in the perinatal heart is necessary to achieve quiescence in this growth-promoting environment. Several studies indicate that the cardiomyocyte growth state in the developing heart correlates with regulated shifts in expression of ECM and integrin receptors and the ability of these matrices to support myocyte growth in vitro.30,31 Thus, we speculate that FRNK may not be necessary in the adult myocardium, because although growth factors such as FGF-2 and IGF-1 continue to be highly expressed, the ECM composition in the adult heart (mainly type I collagen) is not growth-permissive. Interestingly, periostin (a component of the ECM that activates β1, 3, and 5 integrins) is also highly expressed in the developing myocardium and is re-expressed in the diseased adult heart and a recent high profile study demonstrated that recombinant periostin was sufficient to induce cell-cycle re-entry in adult cardiomyocytes both in vitro and in vivo.32 These data underscore the importance of integrin signaling in the regulation of cardiomyocyte cell cycle and support the notion that FAK activity may play a critical role in this response.

Our new studies also reveal that FAK does not merely have a cyto-architectural role in the adult myocardium, but has a key enzymatic function to transmit pressure-induced compensatory progrowth signals. Interestingly, most known genetic mutations leading to familial hypertrophic cardiomyopathy (HCM, which resembles concentric compensatory hypertrophy) are attributable to point mutations in sarcomeric proteins,33 and because these structural molecules are involved in the generation of force that is ultimately transmitted to the cell membrane and ECM, it will be interesting to determine whether FAK can also act to sense the altered load imparted by these mutant muscle fibers. We speculate that FAK might be aberrantly activated in the hearts of mice carrying hypertrophic cardiomyopathy mutations and that FRNK expression might attenuate the extent of hypertrophic remodeling observed. (See the online data supplement for further discussion regarding the necessity of FAK activity for pathological but not anabolic growth of the heart.)

In summary, we show that FRNK expression increases in the myocardium at birth and that FRNK has the capacity to dampen cardiomyocyte proliferation by enhancing activity of the cell cycle inhibitor p38. FRNK−/− mice were recently generated, and these mice were reportedly born in the expected Mendelian frequency and showed no gross phenotype.34 However, our studies indicate that a specific evaluation of cardiac growth in these mice is warranted. Additionally, because we provide evidence that FAK provides cues to neutralize signals responsible for cell cycle restriction, it will also be of interest to determine whether targeted FAK activation can induce postmitotic cardiomyocyte cell cycle reentry.

Sources of Funding

This work was supported, in part, by National Heart, Lung, and Blood Institute grants HL-081844 (to J.M.T.), HL-071054 (to J.M.T.), and HL070953 (to C.P.M.) and American Heart Association Grants 0355776U (to J.M.T.) and 0555476U (to C.P.M.).

Disclosures

None.

References


Transient Expression of FRNK Reveals Stage-Specific Requirement for Focal Adhesion Kinase Activity in Cardiac Growth
Laura A. DiMichele, Zeenat S. Hakim, Rebecca L. Sayers, Mauricio Rojas, Robert J. Schwartz, Christopher P. Mack and Joan M. Taylor

Circ Res. 2009;104:1201-1208; originally published online April 16, 2009; doi: 10.1161/CIRCRESAHA.109.195941
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/104/10/1201

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2009/04/17/CIRCRESAHA.109.195941.DC1
SUPPLEMENTAL METHODS

Antibodies and Reagents. The FAK, and ERK2 (1B3B9) antibodies were purchased from Upstate Biotechnology, Inc. The phosphor-specific and total ERK1/2 (p42/p44) antibodies, phosphor-specific and total p38 antibodies and monoclonal Myc antibody were each purchased from Cell Signaling. The GFP (AV polyclonal) antibody was purchased from Clontech. The phosphospecific tyrosine-397 antibody was purchased from Biosource. The monoclonal M2 flag antibody was purchased from Sigma-Aldrich. The cardiac troponin T monoclonal antibody (13-11) was a gift from Nadia Malouf (UNC, Chapel Hill). Anti-BrdU-biotin was purchased from AbCam and the p27kip antibody was purchased from Santa Cruz. Unless specified, antibodies were used at 1:1000 dilution for Western analysis.

Generation of myc-FRNK Mice. A 1.4kb fragment of myc-FRNK was amplified out of the pRK5myc-FRNK vector to incorporate 3’ and 5’ NotI sites with an internal Kpn site\(^1\). This fragment was then ligated into a vector containing a cassette in which a 1700 bp fragment of the β–actin promoter was fused to cDNA containing GFP flanked by loxP sites. The entire fragment was linearized, and submitted to the Animal Models Core Transgenic Facility at the University of North Carolina for pronuclear microinjection into 0.5 day fertilized embryos. Mice were backcrossed to C57/Black6 for 8 generations prior to subsequent breeding to Nkx2.5\(^{Cre}\) and \(MLc2v^{Cre}\) knock-in mice.\(^2,3\)

Myocyte cell isolation, culture, infection, and treatment: Ventricular cardiomyocytes were isolated from neonatal rats (0-7 days old) by trypsin and collagenase digestion and purified as described previously\(^1\). The cells were resuspended in DMEM:Media 199 (4:1)
containing 10% fetal calf serum and 1% penicillin-streptomycin and plated on tissue culture plastic for two consecutive 1-h periods to remove non-cardiomyocyte cells and to obtain cultures with >95% myocytes. The cardiomyocytes were then plated on tissue culture dishes pre-coated with FN or COL (10 ug/ml) as indicated at a density of $1 \times 10^6$ cells/ml. For adenoviral infection, cells were infected with replication-defective Ad5-GFP, GFP-FRNK, or constitutively active Mek1 (Ser217/221Glu; Seven Hills Bioreagents) at a concentration of 10 m.o.i. for the indicated times. For biochemical assessment of MapKinase and/or AKT activity, cells were incubated in media containing 100μM BrdU (to reduce proliferation of contaminating fibroblasts) and infected with GFP- or GFP-FRNK virus overnight in serum-containing media. Cells were serum-starved overnight and treated for the indicated time points with IGF-1 (10 nM) or FGF-2 (100ng/ml).

**Mouse genotyping:** DNA isolated from tail snips or tissues was subjected to PCR analysis using primers specific for the myc-FRNK transgene (5’-CGCGGTACCATTGAGAATGAGAATTGAGAAGTGAG-3’ and 5’-CGCCTCGAGGCTGAGGAGAAGCTGATC-3’). and for Cre (5’-ACCCTGACCCAGCAAGAGAG-3’ and 5’-CTAGAGCGCTTTTGCACGTTC-3’).

**Histological Analysis and Immunohistochemistry.** Paraffin-embedded hearts were sectioned into 8 μm slices and stained with Masson’s Trichrome stain (Sigma-Aldrich), to assess overall morphology and presence of fibrosis, and hematoxylin and eosin for overall morphology. Following the block of endogenous peroxidases with 0.3% H₂O₂ in methanol, sections were permeabilized with phosphate buffered saline containing 3% BSA w/ 0.2% Triton-X and then incubated with cardiac troponin T (1:200) or GFP
antibody in 1%BSA/PBS overnight at 4 degrees C. The sections were then washed with TBST (TBS plus 0.05% Triton-X), incubated with Texas Red- conjugated donkey anti-mouse antibody or horseradish peroxidase conjugated anti-mouse antibody for 90 min. at room temperature, rinsed, and mounted with Vectamount (Vector Labs). Cell area measurements were conducted on TRITC-Lectin (Sigma-Aldrich) stained cross sections of the compact zone of the heart muscle using Image J software (NIH).

**Apoptosis and Cell Proliferation Assays.** Apoptosis was assessed on paraffin-embedded tissue sections using a DNA fragmentation detection kit, FragEL™ DNA (Calbiochem) according to the manufacturer’s protocol. Cardiac cell proliferation was determined by BrdU labeling which was achieved by administering 100 mg/Kg of BrdU (Sigma) to pregnant mice intraperitoneally. Embryos were harvested 4 hours later, fixed, and embedded in paraffin. Apoptosis and cell proliferation were quantified in vivo by scoring the number of FragEL- and BrdU-positive nuclei per unit area using NIH Image J software.

For analysis is isolated cardiomyocytes, BrdU (10μM) was incubated in serum-containing medium for 48 hr prior to fixation. BrdU incorporation was detected by immunohistochemistry using a biotin-labeled anti-BrdU antibody and fluorophore-conjugated streptavidin following a one hr treatment with 2M HCl and neutralization in 0.1M sodium borate buffer.

**Western Blotting.** Tissues were lysed in modified radioimmune precipitation assay buffer (50 mM Hepes, 0.15 M NaCl, 2 mM EDTA, 0.1% Nonidet -40, 0.05% sodium deoxycholate, pH7.2) containing 1 mM 4-(2-amnionethyl)benzenesulfonfyl fluoride hydroxychloride, 0.02 mg/ml aprotonin containing 5% Triton-X. Proteins were boiled in
a sample buffer and resolved using SDS-PAGE and transferred to nitrocellulose. Immunoblotting was performed using a 1:1000 dilution of the appropriate primary antibody. Blots were washed in Tris-buffered saline, pH 7.4 plus 0.05% Triton-X, followed by incubation with either horseradish peroxidase-conjugated protein A-Sepharose (Amersham Bioscience) or horseradish peroxidase-conjugated rabbit anti-mouse antibody (Amersham Biosciences) at a 1:2000 dilution. Blots were visualized after incubation with chemiluminescence reagents (ECL, Amersham Biosciences).4

**Quantitative RT-PCR.** Ventricles were harvested from end stage mice, homogenized, and RNA was extracted using Trizol reagent (Invitrogen). Each sample of RNA was diluted to 50 ng/μl in DEPC treated H2O. Each 30 μl RNA reaction mixture contained 0.5 μl of 0.1 μg/μl of primer, 1 μl of 20 μmol of probe 15μl of AB Gene one step master mix, and 0.1μl of RT enzyme. The primers and probes used were as follows: 18s forward AGAAACGGCTACCACATCCA, 18s reverse CTCGAAAGAGTCCTGTATTGT, 18s probe TET-AGGCAGCAGGCAGGAATTAC-TAMARA, c-Fos forward CAGTCAGAGAAGGCAAGGCA, c-fos reverse TCCTCTCTGTAATGCACCAG, c-Fos probe FAM-CATCCAGACGTGCCACTGCCCGA-TAMARA. The real time-RT-PCR was performed using the ABI prism 7500 Sequence Detection System (PE Applied Biosystems) in 96-well plates under the following conditions: 30 minutes at 48 ºC, 10 minutes at 95 ºC, 40 cycles of 95 ºC for 15 seconds, and then 60 ºC for one min.

For semi-quantitative RT-PCR analysis, each 20μl reaction contained 250ng of ventricular RNA from end stage mice, 4μl reaction mix and 1μl reverse transcriptase using the iScript cDNA synthesis kit (BioRad). Each reaction was performed under the following conditions: 5 minutes at 25 ºC, 30 minutes at 42 ºC, followed by 5 minutes at
85 °C. Each 50 µl PCR reaction mixture contained 2.5 µl of 10µM of primer, 4µl of dNTP mixture, 5µl of reaction buffer and 0.5 µl of TaKaRa Ex Taq enzyme. The reaction was performed under the following conditions: 5 minutes at 94 °C, 29 cycles of one minute at 94 °C, one minute at 61 °C, 2 minutes at 72 °C, and a final extension of 10 minutes at 72 °C. The primers used were as follows: Bax forward GCGTCCACCAAGAAGCTGAG, Bax reverse ACCACC CTGGTCTTGGATCC, Bcl-2 forward TGTGGCCTTCTTTGAGTTCG, Bcl-2 reverse TCACTTGTGGCCCAGGTATG.

**Transthoracic Echocardiography.** Echocardiograph measurements were taken at the indicated time points using previously described methods. Briefly, following light sedation with isoflurane, hair was removed from the chest and abdomen, and the mice were placed on a water-jacketed, warmed table in the left lateral decubitus position for imaging. A Visualsonic Ultrasound System (Vevo 660) ultrasound machine containing a 30 MHz variable frequency pediatric probe with a 1 cm offset was used to capture the echocardiogram. Standard long axis and short axis M-mode views were recorded when the mouse possessed a target heart rate between 450 and 650 beats per minute. End-diastolic and end-systolic interventricular septum (IVSd, IVSs), posterior wall thickness (PWTd, PWTs) and left ventricular internal diameters (LVEDD, LVESD) were calculated and averaged from three consecutive contractions using Visual Sonics software. Percent fractional shortening was calculated using: %FS=(LVEDD-LVESD)/LVEDD (x 100).

**Biomechanical stress.** Mechanical stress was imposed on the left ventricle using the minimally invasive aortic banding as previously described. Briefly, 20-25 g female
mice were anesthetized (sodium pentobarbital 0.06mg/gr IP), a small incision was made at the suprasternal notch and through the proximal portion of the sternum. Once the aortic arch was visualized, a 6.0 silk suture was placed under the aorta and tied tightly around the vessel using a blunt 27-gauge needle to establish the diameter of the ligature. Sham-operated animals underwent an identical procedure except for lack of ligature placement. The skin was closed and the anesthetized animals were monitored on a heating pad until they recovered. The entire banding procedure lasted approximately 20 min per mouse. Effectiveness of the procedure was monitored by measuring the pulsatility indices before and after banding by Doppler imaging. An average 30-40 cm/sec rise in peak velocity (from a 65-75 cm/sec baseline) in the right carotid with a concomitant decrease in the peak velocity of the left carotid was observed with no significant difference in the gradient between experimental mice and genetic controls. At end stage, the mice were euthanized by CO₂ inhalation and body weight was measured. Following perfusion of the heart with phosphate buffered saline, the hearts and lungs were excised, weighed, and tissues were processed for RNA, protein, or histological analysis as described in detail below. All animal use procedures have been reviewed and approved by the University of North Carolina’s Animal Use and Care Committee.
SUPPLEMENTAL RESULTS

Transient expression of cardiac FRNK

Although the origin of the two forms of FRNK (i.e. 41 and 43 kDa) apparent on the immunoblot in Fig 1A is not completely understood; they could be due to post-translational modifications as previous studies have determined that FRNK can be modified by Ser phosphorylation \(^7\). Interestingly, certain SMC-restricted proteins such as SM22 and SM\(\alpha\)-actin as well as a subset of skeletal muscle transcripts are also transiently expressed in the myocardium, but these proteins, unlike FRNK are most abundant in embryonic hearts between the stages of pre-contractile cardiomyoblast and cardiomyocyte differentiation (see Fig 1A and ref\(^8\)). The finding that FRNK expression was not simply induced during a stage of cardiac development when myocytes express several genes common to both smooth and striated muscle is consistent with our recent studies indicating that FRNK is expressed in an SRF/CArG independent fashion \(^9\).

Generation of FRNK\(^{loxP}\) Mice.

Myc-FRNK was cloned downstream of a region of the \(\beta\)-actin promoter that drives high level expression in all tissues. However, a GFP reporter followed by a transcriptional ‘stop’ site, flanked as a unit by loxP sites, was inserted upstream of the transgene (hereafter referred to as FRNK\(^{loxP}\)) allowing for controlled Cre-dependent expression of myc-FRNK (Fig. 2a, Online Fig. I a).

Characterization of FRNK\(^{Nkx2.5}\) Mice.

While we did observe decreased FAK activity in FRNK\(^{Nkx2.5}\) hearts, no significant difference was observed in the expression levels of the FAK homologue, Pyk2 in lysates from control and FRNK\(^{Nkx2.5}\) hearts (Online Fig. III). Although a modest increase in Pyk2
activity was observed following FRNK expression (not shown), this response was clearly not sufficient to compensate for the loss of FAK activity.

Since the \textit{nkx2-5} promoter drives Cre expression in the pharyngeal endoderm and ectoderm and can induce recombination in tissues derived from these structures, we examined several additional organs histologically to evaluate potential developmental abnormalities that could lead to lethality of FRNK\textsuperscript{\textit{Nk25}} embryos \textsuperscript{3}. There were no overall gross or histological differences observed in the tongue, spleen, and thymus compared to genetic controls at E14 (Online Fig IV). FRNK\textsuperscript{\textit{Nk25}} embryos at E14.5 also exhibited normal craniofacial development and no indication of cleft palate (data not shown).

We used semi-quantitative RT-PCR to measure cardiac and SMC marker gene expression in FRNK\textsuperscript{\textit{loxP}} and FRNK\textsuperscript{\textit{nks2-5}} hearts at E13.5. We did not observe any significant differences in dHand, eHand, TBX1, TBX5, Mef2c, SM22, and SM\alpha-actin (data not shown), indicating that FRNK expression did not have a major impact on the cardiac differentiation program. Furthermore, ex utero M-mode echocardiographic analysis of E13.5 FRNK\textsuperscript{\textit{loxP}} and FRNK\textsuperscript{\textit{nks2-5}} hearts revealed similar heart rates and contractile performance, indicating appropriate maturation of FRNK-expressing cardiomyocytes in the developing heart (data not shown). No significant difference in apoptosis was observed between E13.5 FRNK\textsuperscript{\textit{Nk25}} and genetic control hearts (Fig. 3d). These findings were further substantiated by similar expression of the apoptotic markers Bcl-2 and Bax between these groups (Online Fig V a), and corroborate our previously published studies, demonstrating that ectopic expression of FRNK in cultured neonatal cardiomyocytes did not affect cell survival \textsuperscript{1}.

\textit{FAK represses p38 but induces ERK/JNK activity}
To further examine the relationship of the p38 pathway to FAK signaling, we employed our conditional FAK-null knockout (MFKO) model in which cardiac-restricted deletion of FAK is achieved at 13 weeks post-natal. Using this model system, we previously showed that cardiac-restricted deletion of FAK in the adult mouse did not affect basal myocardial function, but significantly attenuated pressure overload-induced hypertrophic growth and ERK activation. As shown in Online Fig VII, p38 activity was elevated at baseline in MFKO hearts in comparison to genetic controls. Moreover the increase in p38 activity normally observed upon adrenergic stimulation was more pronounced in the MFKO hearts. In contrast, JNK activity was decreased in both basal and agonist-injected MFKO hearts (a result that paralleled the effects of FAK deletion on ERK activity in this model). Taken together, these results indicate that FRNK expression likely dampens cardiomyocyte proliferation by relieving FAK-dependent repression of p38.
SUPPLEMENTAL DISCUSSION

Why is FAK activity necessary for cardiomyocyte proliferation and pathological hypertrophy but dispensable for anabolic growth? Although the role ERK plays in the regulation of anabolic hypertrophic growth is not clear, it is possible that the low levels of ERK activity present in the FRNK^{Mlc2v} hearts is permissive for slow anabolic growth, but is below the threshold required for the induction of proliferation or rapid hypertrophic growth induced by pathological signals. Alternatively, it is possible that normal anabolic growth is regulated by FAK-independent signals. A common downstream pathway elicited by activation of these receptors involves the phosphatidylinositol 3-kinase (PI3K)-AKT-p70S6K protein synthesis pathway. Notably, expression of a constitutive active form of PI3K induces anabolic growth of the heart, while cardiac-restricted expression of a dominant-negative form of PI3K led to smaller hearts accompanied by reduced cell size^{10-13}. Active AKT on the other-hand has been shown to promote cardiomyocyte cell growth or cell proliferation dependent on the locale of activation (cytoplasmic versus nuclear respectively) ^{14-16}. Although FAK has been linked to PI3K signaling in other cell types, we found that neither extracellular matrix nor expression of FRNK altered IGF-1 stimulated activation of AKT in isolated cardiomyocytes, indicating that FAK does not regulate activity of this synthetic pathway, possibly explaining the lack of effect of FRNK expression on anabolic growth.

Interestingly, although we have not observed detectible levels of FRNK in adult rodent hearts under basal conditions, a separate group detected a FRNK-like peptide in the adult myocardium of Wistar-Kyoto rat hearts and showed that the levels of this peptide were increased 3-5 fold in failing hearts from a spontaneously hypertensive line
Although it will be necessary to measure FRNK-specific mRNAs in these models to confirm that the reported peptides are indeed due to autonomous expression of FRNK and were not generated by caspase cleavage of FAK, these data may implicate an additional function for FRNK in aged and/or diseased hearts.
Supplemental Figure Legends

**Online Figure I.** Characterization of FRNK$^{\text{loxp}}$ transgenic mice.  

**a,** Cos7 cells were transfected with the FRNK$^{\text{loxp}}$ construct and infected with Cre adenovirus for the indicated times. Cells were lysed before treatment (time 0), and the indicated time points after infection and Western analysis was performed using anti-Myc and anti-GFP antibodies.  

**b,** Various tissues were harvested from wildtype (wt) and FRNK$^{\text{loxp}}$ transgenic (tg) mice. Western analysis was performed using anti-GFP, anti-Myc and anti-ERK antibodies (loading control). Cos7 cells transfected with Myc-FRNK was used as a positive control (+).

**Online Figure II.** Temporal expression levels of myc-FRNK in FRNK$^{\text{Nkx2.5}}$ and FRNK$^{\text{Mlc2v}}$ hearts relative to endogenous FRNK. Densitometric quantification of Western blot analysis for FRNK levels in hearts from the indicated genotypes. Data are normalized to levels of endogenous FRNK detected at E=12.5. n=3-5/genotype/time point.

**Online Figure III.** Expression level and activity of Pyk2 in hearts from control and FRNK$^{\text{Nkx2.5}}$ mice. Cardiac lysates from E13.5 embryos were immunoblotted with Pyk2, and ERK 2 antibodies (loading control).

**Online Figure IV.** Non-Cardiac Nenk2.5-expressing tissues exhibit normal gross morphology in FRNK$^{\text{nks2.5}}$ mice. Hematoxylin and eosin stained sections of spleen (E14.5), thymus (E14.5) and tongue (E13.5) from FRNK$^{\text{loxP}}$ and FRNK$^{\text{Nkx2.5}}$ embryos.

**Online Figure V.** Expression of Bcl2, Bax, and c-fos in hearts from control and FRNK$^{\text{nks2.5}}$ mice. RNA was extracted from Controls and FRNK$^{\text{Nkx2.5}}$ hearts at E13.5 and
processed for semi- or quantitative-RTPCR a, (left) sq-RTPCR analysis of Bax and Bcl-2 mRNA levels in control and experimental mice: FRNKloxp (L), Nkx2.5Cre (C) and C57bl6 (Wt). Right panel shows densitometric quantification of message levels. b, qRTPCR using the c-fos and 18s probes and primers detailed in the Material and Methods section. c-fos levels were normalized to 18s and presented as fold over control +/- SEM (n >5, * P=0.05).

Online Figure VI. Quantification of FRNK-dependent modulation of p38, ERK, and AKT levels in isolated cardiomyocytes. Western blots from 3-4 separate experiments including representative blots shown in Fig 5c, Fig 5d, and Fig 6b were quantified by densitometry and are presented as mean values +/- SEM.

Online Figure VII. FAK inactivation enhances basal and adrenergic agonist-induced p38 activity. Genetic control and MFKO mice were injected with PBS or an adrenergic cocktail into the intraperitoneal cavity. Mice were sacrificed 7 min later and cardiac lysates were probed using anti-FAK, phospho JNK, phospho p38 or total ERK antibodies. Western data are representative of at least 3 mice per condition. Note, these lysates are identical to those used to assess FAK levels and ERK activation in our previous publication 6.

Online Figure VIII. Constitutively active MEK1 induces a marked increase in hypertrophic growth in FRNK-expressing cardiomyocytes. Rat neonatal cardiomyocytes (P0) were isolated, plated on FN and infected with GFP- or GFP-FRNK adenovirus (10 m.o.i.) and treated with vehicle, SB203580 (10 μM), UO126 (10 μM) or MEK1 adenovirus (10 m.o.i.) as indicated. a, Cells were incubated with BrdU (10 μM) for 48 hr (in serum media) prior to immunohistochemical processing using anti-BrdU and
anti cardiac Troponin T (cTnT) antibodies to identify proliferating cardiomyocytes.  

Western analysis reveals levels of GFP, FRNK, and active ERK in different treatment groups.  Note similar levels of GFP and FRNK expression in all treatment groups in which cells were maintained in serum (SM), while lower levels of each were observed under serum-free (SF) conditions.  Constitutively active Mek1 expression resulted in significant induction of ERK activity in GFP and FRNK-expressing cells.  

Cells were stained with phalloidin to detect serum-dependent increase in cardiomyocyte cell size and organization of sarcomeric actin.  

Cardiomyocyte cell area (2-dimensional) was determined using Image J software and is presented as units relative to GFP expressing cells maintained under serum-free conditions.


<table>
<thead>
<tr>
<th>FRNK&lt;sup&gt;nkx2.5&lt;/sup&gt;</th>
<th>E10.5</th>
<th>E11.5</th>
<th>E12.5</th>
<th>E13.5</th>
<th>E14.5</th>
<th>E16.5</th>
<th>E18.5</th>
<th>3wks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13/65</td>
<td>15/53</td>
<td>25/128</td>
<td>101/476</td>
<td>6/38</td>
<td>1/44</td>
<td>0/14</td>
<td>0/63</td>
</tr>
<tr>
<td>Percentage</td>
<td>20%</td>
<td>26%</td>
<td>19%</td>
<td>21%</td>
<td>16%</td>
<td>2%</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>FRNK&lt;sup&gt;Mic2v&lt;/sup&gt;</th>
<th>3wks</th>
<th>1yr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>95/352</td>
<td>26/106</td>
</tr>
<tr>
<td>Percentage</td>
<td>27%</td>
<td>25%</td>
</tr>
</tbody>
</table>

DiMichele et. al. Online Table I
### Baseline Echocardiography in FRNK$^{Mlc2v}$ and Control mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FRNK</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDD (mm)</td>
<td>2.77 ± 0.11</td>
<td>2.82 ± 0.13</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>1.45 ± 0.14</td>
<td>1.60 ± 0.16</td>
</tr>
<tr>
<td>LVPWTd (mm)</td>
<td>0.90 ± 0.05</td>
<td>0.91 ± 0.08</td>
</tr>
<tr>
<td>LVPWTs (mm)</td>
<td>1.36 ± 0.09</td>
<td>1.25 ± 0.12</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>1.00 ± 0.04</td>
<td>1.01 ± 0.05</td>
</tr>
<tr>
<td>IVSs (mm)</td>
<td>1.48 ± 0.05</td>
<td>1.45 ± 0.06</td>
</tr>
<tr>
<td>%FS</td>
<td>50.00 ± 3.06</td>
<td>45.48 ± 3.69</td>
</tr>
<tr>
<td>%EF</td>
<td>79.9 ± 3.3</td>
<td>75.2 ± 3.5</td>
</tr>
<tr>
<td>HR</td>
<td>515.02 ± 14.65</td>
<td>538.76 ± 18.03</td>
</tr>
</tbody>
</table>

%FS, percent fractional shortening; HR, heart rate; d, diastole; s, systole. Baseline Control n=26, Baseline FRNK$^{Mlc2v}$ n=19.
a. MycFRNK

AdCre (hr)

MycFRNK

GFP

b. Aorta Brain Heart Lung

GFP

Stomach Bladder Intestine Sk. Mus.

GFP

MycFRNK

ERK

DiMichele et. al. Online Figure I
Endogenous FRNK (nor)
DiMichele et. al. Online Figure IV
a. 

<table>
<thead>
<tr>
<th>FRNK&lt;sub&gt;nkx2.5&lt;/sub&gt;</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L L L Cre Cre Wt Wt</td>
</tr>
<tr>
<td>Bax</td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td></td>
</tr>
</tbody>
</table>

b. 

c-fos message (relative to β-actin)

DiMichele et al. Online Figure V
DiMichele et al. Online Figure VI

**a.**
```
pERK/ERK levels (relative to GFP)
```

**b.**
```
p-p38/p38 levels (relative to GFP)
```

**c.**
```
pAKT/AKT levels (relative to GFP)
```
Vehicle Adrenergic Cocktail

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>C</th>
<th>N</th>
<th>N</th>
<th></th>
<th>C</th>
<th>C</th>
<th>C</th>
<th>N</th>
<th>N</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pp38</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pJNK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FAK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ERK2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DiMichele et. al. Online Figure VII
DiMichele et al. Online Figure VIII